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TITLE: Can Diabetes Change the Intrinsic Subtype Specificity of Breast Cancer ?

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| <p>Luminal type A and type B breast cancers represent estrogen receptor alpha (ER<math>\alpha</math>)-positive breast cancers with luminal type A associated with better prognosis. A transcription factor network comprising GATA-3, FOXA1 and ER<math>\alpha</math> dictates estrogen (E2) dependence of luminal type A breast cancer. Signaling molecules that disrupt this network may play a role in E2-independence. T-bet is a major negative regulator of GATA-3. As the expression of some of the above factors are controlled by insulin, the objective of this study was to investigate the ability of insulin, as in type II diabetes, to disrupt GATA-3:FOXA1:ER<math>\alpha</math> network. Insulin induced the expression of T-bet in MCF-7 breast cancer cells and MCF-7 cells engineered to overexpress T-bet (MCF-7-T-bet) were anti-estrogen resistant in the presence of insulin. E2-inducible expression of XBP-1, an ER<math>\alpha</math>:FOXA1:GATA3 downstream target, was attenuated in MCF-7-T-bet cells compared to parental cells suggesting disruption of this hormonal network by T-bet. MCF-7 cells that have acquired resistance to anti-estrogens overexpressed T-bet and expressed lower levels of FOXA1 compared to parental cells. Importantly, Oncomine database search revealed T-bet overexpression in a subgroup of ER<math>\alpha</math>-positive breast cancers, suggesting a role for T-bet in modulating ER<math>\alpha</math> activity in a subset of ER<math>\alpha</math>-positive breast cancers. MCF-7 -xenograft studies with or without experimentally induced diabetes are currently underway to recapitulate in vitro observations in an in vivo setting.</p> |                  |                          |                                     |   |   |
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**Introduction:** Breast cancer has been classified into five intrinsic subtypes with variable prognostic significance based on gene expression profiling studies: Luminal type A (good prognosis), Luminal type B, Her2-positive, Normal-like and Basal-type (worst prognosis group) (1). Luminal type A breast cancers differ from Luminal type B with respect to levels/ activity of estrogen receptor alpha (ER $\alpha$ ) and possibly on their dependency on estrogen for survival (1). In addition, another transcription factor called FOXA1 modulates the function of ER $\alpha$  and is specifically expressed at higher levels in Luminal type A cancers (2, 3). Higher FOXA1 expression associated with good prognosis, a characteristic similar to Luminal A cancers (3). As FOXA1 expression and activity are negatively regulated by Insulin and tumor necrosis factor (TNF), respectively (4), the question is whether metabolic/stress disorders unrelated to breast cancers that lead to elevated serum Insulin and TNF alter the function of ER $\alpha$ :Estrogen:FOXA1 network and thus promote progression of Luminal type A cancers from hormone-dependent to hormone-independent phenotype or to Luminal type B subtype.

A network comprising ER $\alpha$ , estrogen, FOXA1 and GATA-3 constitutes a major morphogenesis and differentiation pathway involving luminal cells of normal breast and is a critical hormonal signaling network in Luminal type A breast cancer (1, 5, 6). In fact, ER $\alpha$ :FOXA1:GATA3 transcription factor network has been dubbed as a cell lineage enriched hormonal network in breast cancer (7). While ER $\alpha$ :estrogen axis has been the major focus of investigation, how metabolic/stress conditions that alter the expression/activity of FOXA1 and GATA-3 change the biology of Luminal type A breast cancers is not known. Our hypothesis is that in breast cancer patients who develop diabetes subsequent to their initial diagnosis of having Luminal type A breast cancers, recurrent tumors may not retain gene expression pattern of Luminal type A subtype because of the effects of Insulin or TNF on FOXA1 expression/activity. As a consequence, ER $\alpha$ :estrogen:FOXA1:GATA-3 network may not be the major signaling network in recurrent tumors and is replaced by ER $\alpha$ -independent proliferation/survival pathway.

### **Body:**

**Objective:** Investigate whether Luminal type A breast cancer cell line MCF-7 implanted into nude mice with experimentally induced type I diabetes (no Insulin but hyperglycemia) or type II diabetes (elevated serum Insulin and hyperglycemia) progress to become Luminal type B or any other intrinsic subtype.

### **Progress on in vitro studies to identify targets of Insulin that may disrupt hormonal network:**

**T-bet is overexpressed in a subset of ER $\alpha$ -positive breast cancers:** GATA-3 was originally characterized as a signaling molecule involved in T cell differentiation (8). Subsequent studies have shown similar role for GATA-3 in differentiation of luminal progenitor cells of the breast (6). T-bet, also called Tbx21, is a negative regulator of GATA-3 activity in T cells (8). Furthermore, T-bet is expressed in the epithelial cells of the endometrium; expression of both GATA-3 and T-bet cyclical in these cells suggesting hormonal regulation of their expression (9). Therefore, we considered the possibility of T-bet regulating GATA-3 activity in breast cancer cells and disrupting GATA-3, FOXA1 and ER $\alpha$ -regulated hormonal network.

The Oncomine database was utilized to determine a correlation between T-bet and ER $\alpha$  expression in primary breast cancer. Although T-bet is expressed at higher levels in ER $\alpha$ -negative breast cancers compared to ER $\alpha$ -positive breast cancers, a subset of ER $\alpha$ -positive breast cancers expressed higher levels of T-bet (Figure 1). T-bet expression was also associated with progesterone receptor (PR) negativity, a subgroup that is known for being resistant to endocrine therapy. This begs the question whether T-bet positivity could be related to endocrine insensitivity in ER $\alpha$  positive tumors.

**Insulin induces T-bet expression in breast cancer cells:** To determine whether T-bet is expressed in breast cancer cell lines and the expression is regulated extracellular signals, we investigated the effects of growth hormone, Insulin, Insulin-like growth factor II, and estrogen on T-bet expression in MCF-7 cells. Insulin but not other factors induced T-bet expression (Figure 2A).

We next examined the effects of Insulin on GATA-3 and FOXA1 expression. If crosstalk between T-bet and GATA-3 is similar in both T cells and breast epithelial cells, Insulin is expected to reduce the expression and/or activity of GATA-3. Consequently, FOXA1 expression should be low in Insulin-treated cells. As expected, Insulin reduced the expression of GATA-3 but failed to have significant effect on the expression of FOXA1 (Figure 2B). These results suggest that Insulin-induced T-bet significantly effects GATA3 expression. Unlike in normal mammary gland, FOXA1 expression in cancer cells may be independent of GATA-3.

Additional ER $\alpha$ -positive breast cancer cell lines were examined for basal and Insulin-regulated expression of T-bet. T-bet expression was markedly higher in BT-474 cells, which is considered as luminal type B cell line (Figure 2C). GATA3 is not expressed in these cells; however, FOXA1 is expressed at high level suggesting GATA-3 independent expression of FOXA1 in these cells. T47-D cells also express T-bet; however, unlike MCF-7 cells, T-bet expression was not regulated by Insulin. Taken together, these results reveal an association between T-bet and GATA-3 expression in breast cancer cells and cell type-specific effects of Insulin in modulating their expression.

**T-bet overexpression in MCF-7 cells leads to altered estrogen and tamoxifen response:** To determine whether T-bet negatively regulates estrogen-inducible expression of specific ER $\alpha$ , GATA3 and FOXA1 target genes, we generated MCF-7 cells overexpressing T-bet (Figure 3A). XBP1 has been suggested as the downstream target of ER $\alpha$ , FOXA1 and GATA3 network as meta-analysis has revealed coexpression of all four of these genes in a significant proportion of breast cancer (10). Binding sites for all three transcription factors are also present in the regulatory region of XBP-1 (2). While XBP-1 expression was readily estrogen-inducible in parental cells, estrogen failed to induce the expression of XBP-1 in T-bet overexpressing cells (Figure 3B). The effect of T-bet on estrogen-inducible expression is gene-specific because estrogen induced Myb and Cyp19 to similar levels in parental and T-bet overexpressing cells.

**T-bet overexpressing cells are less sensitive to tamoxifen in the presence of Insulin:** To further evaluate the effect of T-bet mediated changes in estrogen response on sensitivity to anti-estrogens, we examined tamoxifen sensitivity of parental and T-bet overexpressing cells. Both cell types were similarly sensitive to tamoxifen in the absence

of Insulin (Figure 4). T-bet overexpressing cells were more responsive to Insulin-mediated growth stimulation and consequently, were less sensitive to tamoxifen. Thus, it appears that Insulin not only induces T-bet expression but also influences T-bet function as evident from enhanced proliferation.

**Anti-estrogen resistant cells express higher levels of T-bet:** We used a clonal variant of MCF-7 cells and the same clone that acquired tamoxifen and Fulvestrant (ICI182,780) resistance (11) to determine whether there is a correlation between anti-estrogen resistance and T-bet expression. Both tamoxifen-resistant and ICI-resistant cells expressed higher levels of T-bet (Figure 5). FOXA1 expression was significantly reduced in these cells compared to parental cells. Thus, anti-estrogen resistance may involve selective upregulation of T-bet and loss of FOXA1 expression. Insulin may contribute to this process by increasing the expression and/or post-translational modification of T-bet.

**Ongoing studies:**

- 1) **Xenograft studies with MCF-7 cells under normal and experimentally induced diabetic condition:** We are waiting for tumors to develop in these animals for analysis.
- 2) **siRNA knockdown to T-bet in parental and tamoxifen resistant cells.** These studies are being performed to evaluate whether some of the observed effects of Insulin and tamoxifen resistance is dependent on T-bet.

**Key Research Accomplishments:**

- ☐ Luminal Type A breast cancers are characterized by a transcription factor signature comprised of GATA-3, ER $\alpha$ , and FOXA1.
- ☐ Treatment with Insulin induces the expression of T-bet and decreases GATA-3 expression in MCF-7 cells.
- ☐ Estrogen fails to induce XBP-1, a ER $\alpha$ :FOXA1:GATA-3 downstream target gene, in MCF-7 cells overexpressing T-bet. These results suggest T-bet mediated loss of Luminal Type A phenotype.
- ☐ Insulin confers tamoxifen resistance in those MCF-7 cells overexpressing T-Bet.
- ☐ Tamoxifen and Fulvestrant resistant cells overexpress T-bet and display disruption of ER $\alpha$ :FOXA1 and GATA-3 network.
- ☐ Disruption of the GATA-3:ER $\alpha$ :FOXA1 transcription network by T-Bet and Insulin provide a mechanism for progression of luminal type A to endocrine treatment resistance.

**Reportable Outcomes: 2 abstracts published and one more is accepted for 2008 San Antonio Breast Cancer Symposium**

**1) Control of Luminal Type A Intrinsic Subtype Enriched Transcription Factor Network by Insulin: Implications of Diabetes on Breast Cancer Subtypes.**  
Harikrishna Nakshatri, Kasi R. McCune, Poornima Bhat-Nakshatri, Mangesh Thorat, and Sunil Badve. Department of Defense Era-of-Hope meeting June 2008, Baltimore

**2) Control of Luminal Type (Lum) A Subtype Enriched Transcription Factor Network by Insulin: Implications of Diabetes on Breast Cancer Classification.**  
Kasi R. McCune, Poornima Bhat-Nakshatri, Mangesh Thorat, Sunil Badve, Harikrishna Nakshatri, ASCO Breast Symposium, Washington, DC, September 2008.

**Conclusions:** ER $\alpha$ , FOXA1 and GATA-3 constitute a key hormonal transcription factor network in Luminal type A breast cancer. Elevated levels of Insulin, as observed in diabetes, can disrupt this network and confer estrogen-independence through upregulation of T-bet, a transcription factor known to negatively regulate GATA-3 expression and/or activity. The above observations, which were generated through studies in vitro, are currently being evaluated in an in vivo model.

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**Appendices:**

Two abstracts.

**Supporting data:**

Figures



General Poster Session C (Board #A11), Sat, 12:00 PM - 1:15 PM

**Prediction of response to neoadjuvant endocrine therapy by gene expression profiles in women with breast cancer.** *S. Kashi-Tanaka, C. Shimizu, K. Yoshimura, M. Andoh, T. Kuno, N. Katsumata, T. Hojo, M. Yoshida, T. Kinoshita, Y. Fujiwara; National Cancer Center Hospital, Tokyo, Japan*

**Background:** The prognostic and predictive ability of the 21-gene recurrence score (RS) is established in patients treated with adjuvant tamoxifen. The aim of this study was to explore whether the clinical response to neoadjuvant endocrine therapy could be predicted by RS or not. **Methods:** Postmenopausal women with estrogen receptor-positive breast cancer received 4-month neoadjuvant endocrine therapy in prospective studies with the same inclusion criteria. RS score was quantified from pretreatment formalin-fixed paraffin-embedded core biopsy specimens. Response to endocrine therapy was measured in two dimensions with calipers before and after neoadjuvant endocrine therapy. **Results:** RS could be measured from the samples of 43 core needle biopsies. Fourteen patients received neoadjuvant tamoxifen and 29 received neoadjuvant anastrozole. Patients with low RS (<18) tended to respond better than those with intermediate and high RS (64% vs. 31% and 31%,  $p$  for trend = 0.11). RS tended to predict response both in tamoxifen-treated (67%, 33%, and 40%) and anastrozole-treated patients (63%, 30%, and 27%). Patients with low-RS also tended to have better relapse-free survival (RFS) than those with intermediate and high RS (5y-RFS; 100% vs. 84%, 73%, respectively). **Conclusions:** Although this study was small in sample size, the results suggested RS may predict response to neoadjuvant endocrine therapy not only with tamoxifen but also anastrozole. These results should be validated in larger studies.

48 General Poster Session C (Board #A12), Sat, 12:00 PM - 1:15 PM

**Control of luminal (Lum) A subtype enriched transcription factor network by insulin: Implications of diabetes on breast cancer classification.** *K. R. McCune, P. Bhat-Nakshatri, M. Thorat, S. Badve, H. Nakshatri; Indiana University School of Medicine, Indianapolis, IN*

**Background:** LumA breast cancers express higher levels of ER $\alpha$  and are associated with better prognosis than LumB. A transcription factor network comprising GATA-3, FOXA1, and ER $\alpha$  is proposed to dictate hormone dependence of LumA cancers. Thus, signaling molecules that disrupt this network may force LumA cancer cells to acquire hormone independence or anti-estrogen (E<sub>2</sub>) resistance. T-bet (Tbx21) is a well established negative regulator of GATA-3. As the expression of some of the above factors is controlled by insulin, the objective of this study was to investigate whether elevated level of insulin, as in type II diabetes mellitus, alters gene expression patterns and anti-E<sub>2</sub> sensitivity of LumA breast cancers by altering this network. **Methods:** The effect of insulin on the expression of ER $\alpha$ , FOXA1, GATA-3, and T-bet was measured in ER $\alpha$ -positive MCF-7 cells by Western blotting. The effect of T-bet on E<sub>2</sub>-regulated gene expression was measured by qRT-PCR analysis of E<sub>2</sub> inducible genes in parental MCF-7 and MCF-7 stably overexpressing T-bet (MCF-7-Tbet). BRDU-ELISA assay was used to measure the effect of E<sub>2</sub>, tamoxifen, and insulin on cell proliferation. The expression pattern of T-bet and its relation to ER $\alpha$  status in primary breast cancers were determined using Oncomine database. **Results:** Insulin induced the expression of T-bet, which was partially reversed by E<sub>2</sub>. ER $\alpha$  and GATA-3 levels were reduced in MCF-7-Tbet cells compared to MCF-7 cells. E<sub>2</sub>-inducible expression of GREB-1 and Myb were lower in MCF-7-Tbet cells compared to MCF-7 cells, although basal expression of these genes was higher in MCF-7-Tbet cells. MCF-7-Tbet but not MCF-7 cells were resistant to tamoxifen in the presence of insulin. Although T-bet expression was observed predominantly in ER $\alpha$ -negative breast cancers, a subset of ER $\alpha$ -positive breast cancers overexpressed T-bet. **Conclusions:** Insulin may change the gene expression pattern through Tbet-mediated disruption of GATA-3, ER $\alpha$ , and FOXA1 hormonal transcriptional network in LumA cancers. Tbet may serve as a marker to identify a subset of LumA cancers that have progressed to hormone independence.

## P28-1: CONTROL OF LUMINAL TYPE A INTRINSIC SUBTYPE ENRICHED TRANSCRIPTION FACTOR NETWORK BY INSULIN: IMPLICATIONS OF DIABETES ON BREAST CANCER SUBTYPES

Harikrishna Nakshatri,<sup>1</sup> Kasi R. McCune,<sup>1</sup> Poornima Bhat-Nakshatri,<sup>1</sup> Mangesh Thorat,<sup>1</sup> and Sunil Badve<sup>2</sup>  
Indiana University-Purdue University, Indianapolis, and <sup>2</sup>Indiana University, Indianapolis

**Background:** Breast cancer is classified into five intrinsic subtypes based on gene expression pattern; luminal type A, luminal type B, ErbB2/Her-2-positive, basal, and normal-like. Luminal type A and type B represent estrogen receptor  $\alpha$  (ER $\alpha$ )-positive breast cancers with luminal type A expressing higher levels of ER $\alpha$  and is associated with better prognosis. Recent studies have identified a specific functional transcription factor network comprising GATA-3, FOXA1, and ER $\alpha$  in normal luminal cells as well as in luminal type A breast cancer that dictates their hormone dependence. Signaling molecules that may disrupt this network and force these cells to acquire hormone-independence are not known. T-bet (Thx21) has been described as a major negative regulator of GATA-3 activity. As the expression and/or activity of some of the above factors are controlled by insulin, the objective of this study was to investigate whether elevated level of insulin, as evidenced in type II diabetes, alters gene expression pattern in luminal type A breast cancers by interrupting GATA-3:FOXA1:ER $\alpha$  network and thus forcing these cancers to acquire nonluminal phenotype and/or hormone-independence.

**Methodologies:** The effect of insulin on the expression of ER $\alpha$ , FOXA1, GATA-3, and T-bet was measured in ER $\alpha$ -positive MCF-7 cells by western blot analysis. The effect of T-bet on estrogen-regulated gene expression was measured by transient transfection assays and stable overexpression of T-bet in MCF-7 cells. Publicly available Oncomine database was used to determine the expression pattern of T-bet and its relation to ER $\alpha$  status in primary breast cancers. Immunohistochemistry was used to determine T-bet expression in normal breast and breast cancer.

**Results:** Insulin induced the expression of T-bet, which was partially reversed by estrogen. In transient transfection assays, T-bet reduced estrogen response element-driven reporter gene expression. ER $\alpha$  and GATA-3 levels were reduced in MCF-7 cells stably overexpressing T-bet suggesting that T-bet reduces GATA-3-dependent ER $\alpha$  expression. Estrogen-inducible expression of estrogen target gene GREB-1 was lower in T-bet overexpressing cells compared to parental cells. Importantly, ER $\alpha$ -negative breast cancers showed higher T-bet expression suggesting mutual antagonism between ER $\alpha$  and T-bet.

**Conclusions:** Insulin may change the gene expression pattern through T-bet-mediated disruption of master cell-type-specific transcriptional network including GATA-3, ER $\alpha$ , and FOXA1 that dictates the phenotype of hormone-dependent luminal type A breast cancer. T-bet may serve as a marker to differentiate FOXA1+/GATA-3+ breast cancers that may have progressed to hormone-independence.

*This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0651.*

## P28-2: FUNCTIONAL RELATIONSHIPS BETWEEN HER2 AND THE LEPTIN SYSTEM IN BREAST CANCER

Eva Surmacz  
Temple University

**Background:** The obesity hormone leptin (Ob) has been implicated in tumorigenesis, especially in the development of breast cancer (BC). The mitogenic, angiogenic, and antiapoptotic activities of Ob are mediated through the leptin receptor (ObR). Data obtained in HEK293T cells engineered to coexpress ObR and the oncoreceptor HER2 suggested that Ob can transactivate HER2 via ObR. To address this putative interaction, we studied whether simultaneous expression of Ob, ObR, and HER2 can occur in human BC. In cellular models, we studied if ObR and HER2 can physically interact and if activation of Ob/ObR is able to transactivate HER2.

**Material and Methods:** The expression of Ob and ObR was evaluated by immunohistochemistry in 59 BCs (31 HER2-positive, 28 HER2-negative). Ob and ObR were classified as positive (at least +) or negative (below +). The relationships among Ob and ObR and the clinicopathological features, that is, grading (G1, G2, and G3), tumor size (diameter in mm), node involvement (positive or negative), vascular invasion (positive or negative), and ER and PgR expression (positive or negative) were analyzed using the Chi square test. The mechanistic relationships between ObR and HER2 were studied by western blotting, co-immunoprecipitation, and immunofluorescence/deconvoluted microscopy in MCF-7 cells that are sensitive to Ob and coexpress both receptors.

**Results:** Ob and ObR were coexpressed in 78% of BC and were correlated in all BCs (p10 mm) node-positive tumors (trends  $p=0.06$  and  $p=0.08$ ). The simultaneous expression of Ob/ObR and HER2 was found in 39% of BCs, but the Ob/ObR system was also frequent in HER2-negative BCs. Ob, ObR, and combined Ob/ObR did not correlate with HER2, grading, VI, and ER/PgR. Using MCF-7 cells, we found that a

fraction of ObR and HER2 are colocalized and can be found in one immunocomplex. We also demonstrated that 100 ng/mL Ob can activate HER2 tyrosine phosphorylation upon 15 min-1 h treatment.

**Conclusions:** Ob and ObR are often coexpressed, and a subset of Ob/ObR-positive tumors exhibits concomitant expression of HER2. In BC cells, HER2 and ObR may physically interact and Ob can cross-activate HER2. Thus, high levels of Ob found in obese patients might lead to the activation of the Ob/ObR/HER2 signaling in BC contributing to the resistance of BC to anti-HER2 treatments.

*This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0603 and The Sbarro Health Research Organization.*

## P28-3: PROLACTIN PROMOTES MAMMARY TUMORIGENESIS SECONDARY TO LOSS OF p53

Kathleen O'Leary and Linda Schuler  
University of Wisconsin, Madison

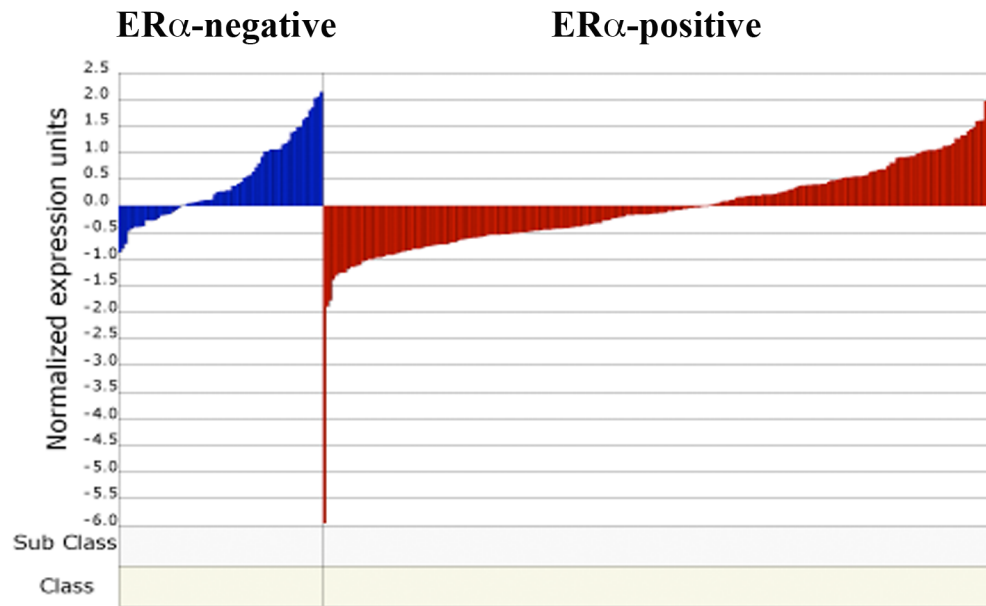
Multiple factors and pathways contribute to mammary tumorigenesis. Two factors that have separately been implicated in breast cancer are the hormone prolactin (PRL) and the tumor suppressor p53. This study has begun to examine the interaction between these factors using two mouse models: p53<sup>-/-</sup> mice made congenic on the FVB/N background and the NRL-PRL transgenic mouse model which employs a non-hormonally regulated, mammary selective promoter to drive expression of PRL in mammary epithelium. Mammary gland transplants were performed on the following genotypes: wildtype, p53<sup>-/-</sup>, NRL-PRL/p53<sup>-/-</sup>, and NRL-PRL/p53<sup>+/+</sup> to circumvent the problem that p53<sup>-/-</sup> mice are prone to multiple tumors. This study demonstrates that increased PRL combined with loss of p53 cooperatively affect mammary tumorigenesis in multiple ways. Tumor latency is decreased. Mean survival of the NRL-PRL/p53<sup>-/-</sup> recipients was 209 days as compared to 247 days for the p53<sup>+/+</sup> recipients. In addition, the tumors appeared to be more aggressive. For the NRL-PRL/p53<sup>-/-</sup> tumors, 5/15 (33%) invaded into the peritoneal cavity. In contrast, this was not observed for the p53<sup>+/+</sup> tumors 0/9 (0%); all were confined to the mammary fat pad. However, NRL-PRL/p53<sup>-/-</sup> and p53<sup>+/+</sup> tumors were similar histologically. Both were highly anaplastic and were identified as either carcinosarcomas, spindle cell tumors or adenocarcinomas, indicating that loss of p53 appears to dictate the tumor histology. Thus, it appears that PRL and p53 pathways interact to promote breast cancer by decreasing latency and increasing invasiveness. Further studies are being done to examine the contribution of genomic instability to this process.

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## P28-4: INHIBITION OF ESTROGEN-INDUCED GROWTH OF BREAST CANCER CELLS BY MODULATING IN SITU OXIDANT LEVELS

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The purpose of our BCRP-funded proposal (BC051097) was to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, i.e., estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle. We previously reported that 17 $\beta$ -estradiol (E2)-induced mitochondrial (mt) reactive oxygen species (ROS) act as signaling molecules. Here we have examined whether antioxidants, N-acetylcysteine and ebelen, overexpression of ROS lowering genes, such as catalase and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA, by real-time RT-PCR, the rate of DNA synthesis by BrdU incorporation, and different phases of cell cycle by flow cytometry. We also determined the morphology and behaviors of cells that overexpress mtSOD, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Our data revealed that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebelen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells that is evident from the lower incorporation of BrdU in siRNA treated cells compared to wild-type cells in the presence of E2. We observed similar results by flow cytometry. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase. Both antioxidant treatment and dextoxification of ROS prevented E2-induced expression of cyclin D1 and pcna, markers of cell proliferation detected by Real time PCR. In cells overexpressed with adenovirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using their siRNA, E2 was not able to produce any colony. Both antioxidants ebelen and N-acetylcysteine produced similar effects. It appears that E2-dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling. Since neither antioxidants nor mito-



**Figure 1**

Figure 1: Oncomine database search revealed overexpression of T-bet in a subset of ERα-positive breast cancers. Overall expression was significantly higher in ERα-negative breast cancer compared to ERα-positive breast cancers ( $p < 0.000001$ ).

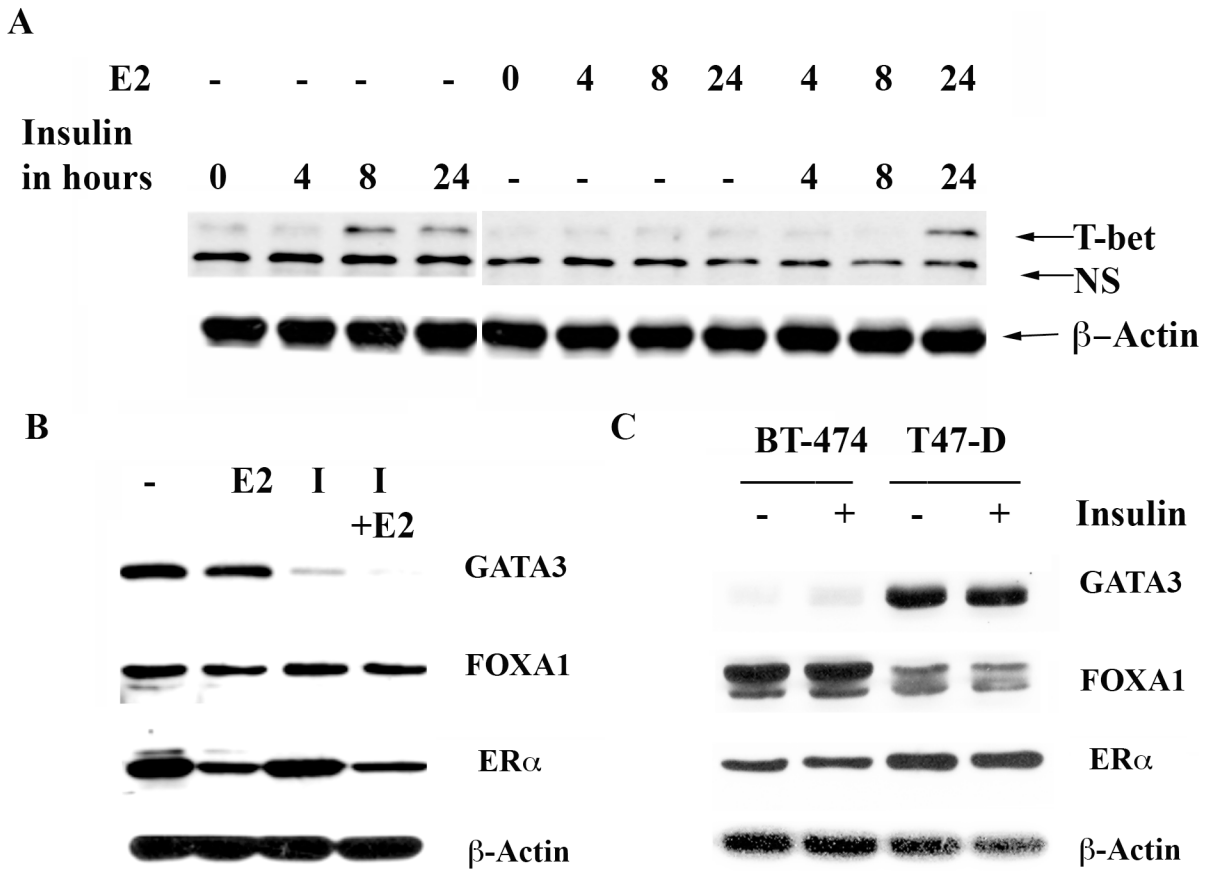
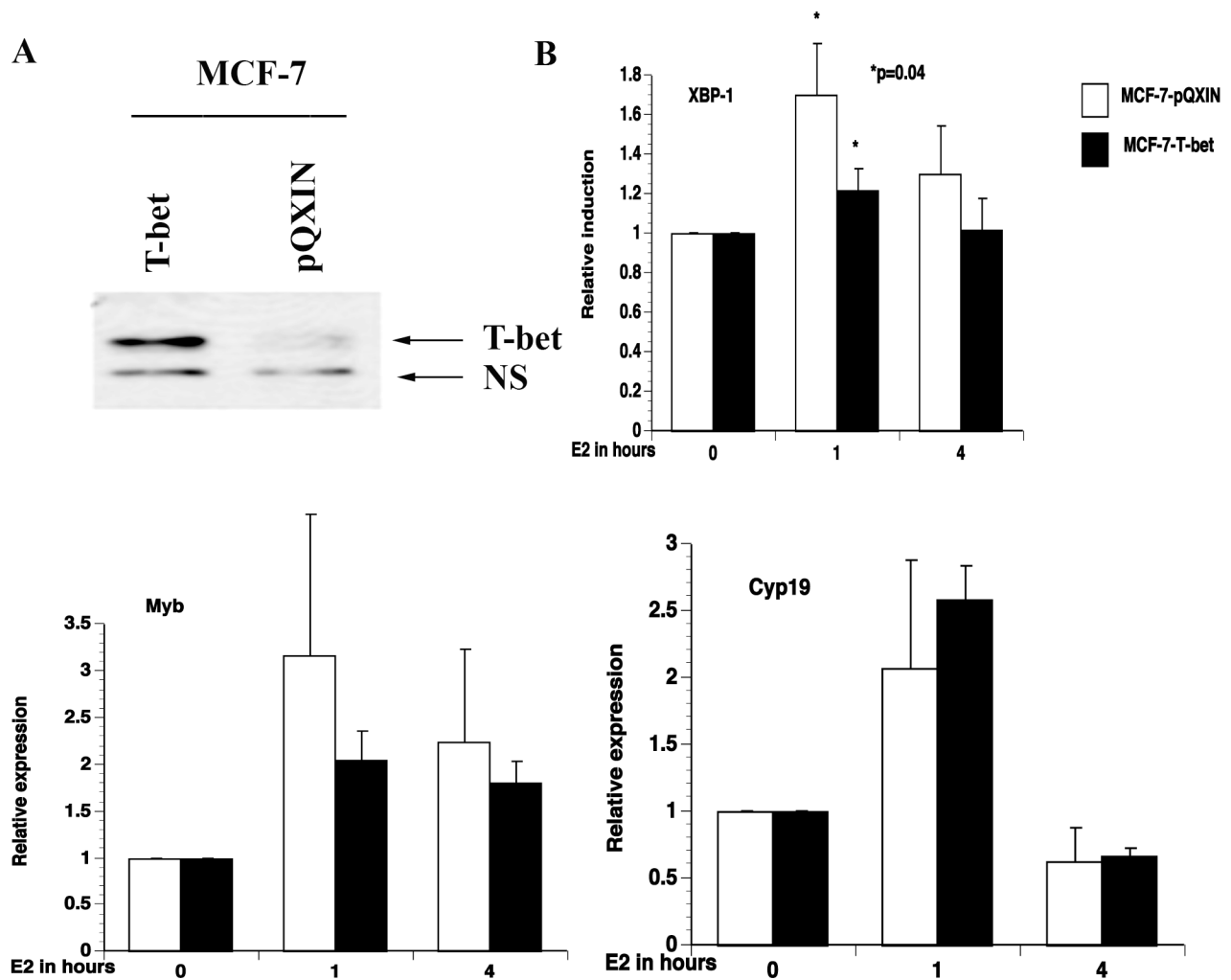


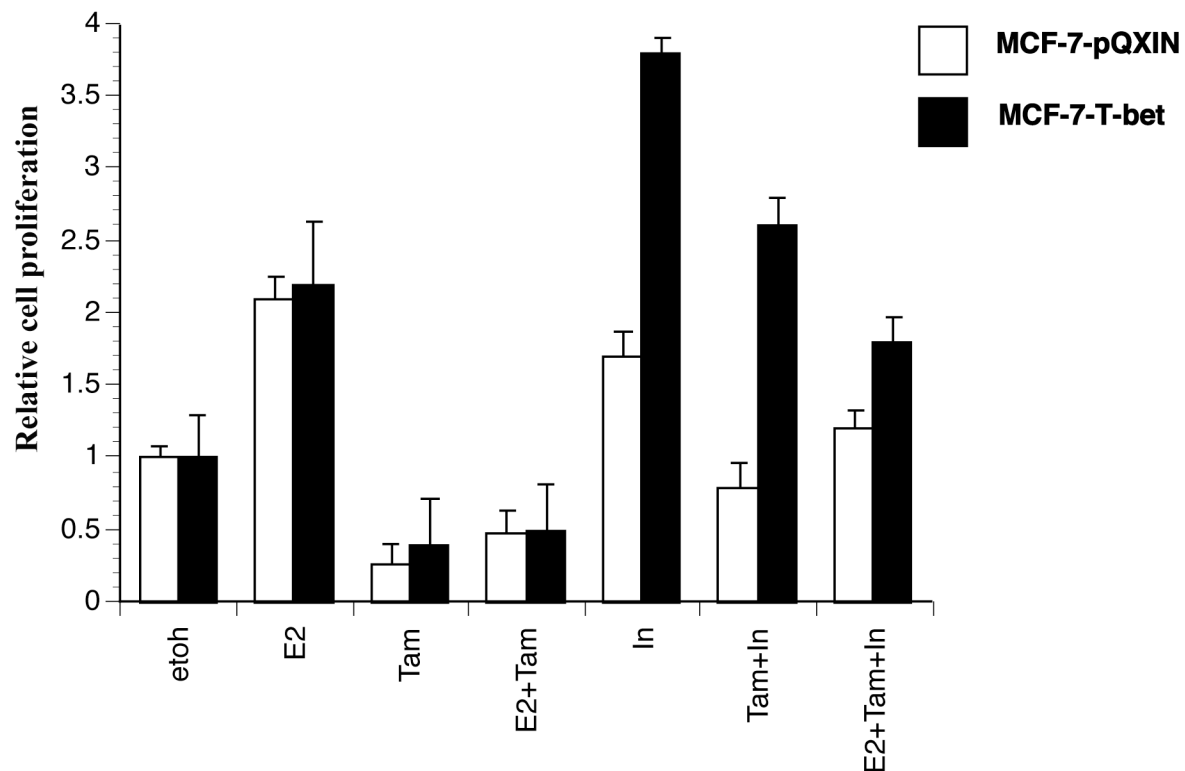
Figure 2: A) Insulin induces T-bet expression in MCF-7 cells. Estrogen reduced Insulin-induced T-bet expression only at 8 hours time point. Cells were treated with 50 nM Insulin and/or  $10^{-10}$  M estrogen. B). Insulin reduces GATA3 expression. Cells were treated with Insulin and/or estrogen for 24 hours as in A and lysates were analyzed for the expression of indicated proteins by Western blotting. C) Expression pattern of T-bet, GATA3 and FOXA1 in T47-D and BT-474 (ER $\alpha$ -positive) cells. Cells were treated with Insulin for 24 hours.

**Figure 2**



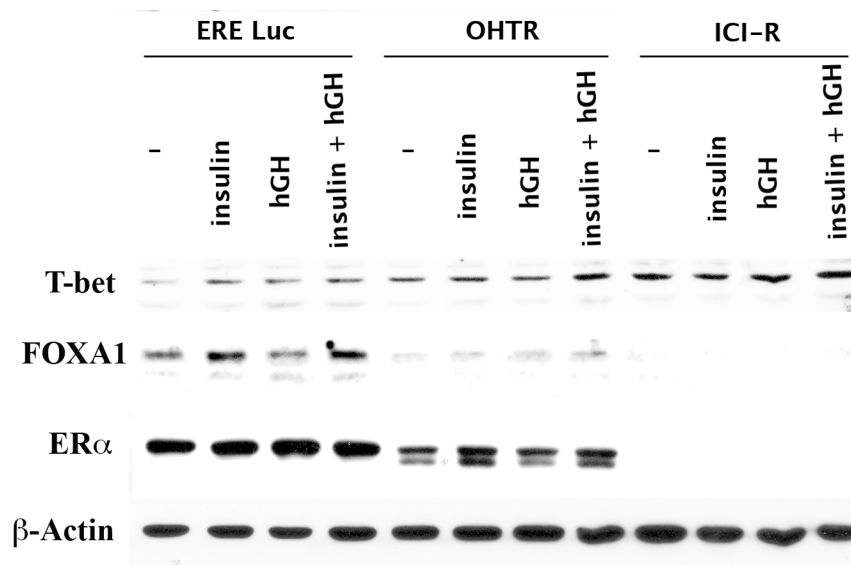
**Figure 3**

Figure 3: A) Generation of MCF-7 cells overexpressing T-bet. T-bet overexpressing cells were generated using a bicistronic retrovirus system. pQXIN corresponds to parental MCF-7 cells retrovirus vector alone. B) T-bet overexpression leads to loss of estrogen-inducible expression of XBP-1 but not Myb or Cyp19. Cells were treated with estrogen for indicated time and mRNAs corresponding to indicated genes were measured by quantitative reverse transcription polymerase chain reaction.



**Figure 4**

Figure 4: T-bet overexpressing cells are growth stimulated by Insulin and are less sensitive to tamoxifen. Cells were treated with indicated reagents for six days and bromodeoxyuridine incorporation-ELISA was used to measure the rate of cell proliferation. Proliferation rate of ethanol (vehicle for estrogen)-treated cells was normalized to one and relative proliferation is presented.



**Figure 5**

Figure 5: T-bet is overexpressed in tamoxifen (OHTR) and Fulvestrant-resistant (ICI-R) resistant derivatives of MCF-7 cells. ERE-Luc corresponds to parental MCF-7 cells but generated from a single clone expressing ERE-luciferase reporter gene (Ref. 11). Therefore, T-bet overexpression and loss of FOXA1 expression are acquired phenotypes during progression to anti-estrogen resistance.